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The MerR-Like Regulator BrlR Impairs *Pseudomonas aeruginosa* Biofilm Tolerance to Colistin by Repressing PhoPQ

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While the MerR-like transcriptional regulator BrlR has been demonstrated to contribute to *Pseudomonas aeruginosa* biofilm tolerance to antimicrobial agents known as multidrug efflux pump substrates, the role of BrlR in resistance to cationic antimicrobial peptides (CAP), which is based on reduced outer membrane susceptibility, is not known. Here, we demonstrate that inactivation of *brlR* coincided with increased resistance of *P. aeruginosa* to colistin, while overexpression of *brlR* resulted in increased susceptibility. *brlR* expression correlated with reduced transcript abundances of *phoP*, *phoQ*, *pmrA*, *pmrB*, and *arnC*. Inactivation of *pmrA* and *pmrB* had no effect on the susceptibility of *P. aeruginosa* biofilms to colistin, while inactivation of *phoP* and *phoQ* rendered biofilms more susceptible than the wild type. The susceptibility phenotype of Δ*phoP* biofilms to colistin was comparable to that of *P. aeruginosa* biofilms overexpressing *brlR*. BrlR was found to directly bind to *oprH* promoter DNA of the *oprH-phoPQ* operon. BrlR reciprocally contributed to colistin and tobramycin resistance in *P. aeruginosa* PAO1 and CF clinical isolates, with overexpression of *brlR* resulting in increased tobramycin MICs and increased tobramycin resistance but decreased colistin MICs and increased colistin susceptibility. The opposite trend was observed upon *brlR* inactivation. The difference in susceptibility to colistin and tobramycin was eliminated by combination treatment of biofilms with both antibiotics. Our findings establish BrlR as an unusual member of the MerR family, as it not only functions as a multidrug transport activator, but also acts as a repressor of *phoPQ* expression, thus suppressing colistin resistance.

Pseudomonas aeruginosa is an opportunistic pathogen associated with chronic colonization of a variety of different human tissues and medical instruments. The ability of *P. aeruginosa* to form biofilms within the airways of individuals with the genetic disease cystic fibrosis (CF) plays an important role in CF-associated chronic infections by allowing the bacteria to withstand host defenses and antimicrobial treatment (1, 2). Biofilms are complex communities of surface-associated microorganisms embedded within a self-produced extracellular matrix (3, 4). Biofilm communities exhibit increased tolerance to antimicrobial agents, often leading to persistent and chronic infections that are difficult to eradicate (5, 6). Overall, biofilms can be up to 1,000 times more resistant to antibiotics than their planktonic counterparts (6). Biofilm tolerance is distinct from resistance mechanisms more commonly associated with planktonic cells, such as acquiring resistance through random mutation or uptake of plasmid-borne resistance markers (7, 8). This high-level tolerance observed in biofilms is thought to be multifactorial, requiring a combination of different mechanisms. Mechanisms contributing to high antimicrobial tolerance in biofilms are thought to include reduced metabolic and growth rates compared to planktonic cells (9–11), the presence of dormant persister cells that are not killed by exposure to antibiotics (12), restricted diffusion of certain types of antimicrobial agents into a biofilm (8, 13, 14), starvation-induced growth arrest (15), and the maturity or developmental stage of the biofilm (10, 16).

Recent evidence further suggested that bacteria within microbial communities employ a specific regulatory mechanism to resist the action of antimicrobial agents. Liao et al. demonstrated that in the human pathogen *P. aeruginosa*, biofilm tolerance is conferred, in part, by BrlR, a member of the MerR family of multidrug efflux pump activators (17, 18). BrlR was found to contribute to the high-level drug tolerance of biofilms formed by *P. aeruginosa* by affecting the MICs of and recalcitrance to killing by bactericidal antimicrobial agents, including tobramycin, tetracycline, chloramphenicol, trimethoprim, and norfloxacin (17). These antibiotics are substrates of multidrug efflux pumps and act by inhibiting either protein synthesis or DNA replication (19). It is thus not surprising that BrlR was recently reported to activate the expression of genes encoding the multidrug efflux pumps MexAB-OprM and MexEF-OprN (18). However, the contribution of BrlR to the tolerance of *P. aeruginosa* biofilms to cationic antimicrobial peptides (CAP) was not investigated.

The mode of action of CAP is based on the loss of the membrane barrier property (20, 21). Cationic antimicrobial peptides pass across the outer membrane by interacting with negatively charged lipopolysaccharide (LPS). The electrostatic interaction competitively displaces the divalent polyanionic cations and partly neutralizes LPS, leading to outer membrane permeabilization in a process termed self-promoted uptake (21). Once peptides have crossed the lipid bilayers, evidence suggests that the peptides partition into the cytoplasmic membrane through hydrophobic and electrostatic interactions, causing stress in the lipid bilayer and, ultimately, loss of the barrier property of the membrane (20, 22, 23). Colistin (also called polymyxin E) is a cationic antimicrobial peptide belonging to the polymyxin group of antibiotics (24). Resistance to cationic antimicrobial peptides such as colistin can be mediated by the addition of positively charged

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TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or description	Source
Strains		
E. coli		
DH5α	λ^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	Invitrogen Corp.
BL21(DE3)	F^- *ompT hsdS*$_B$ ($r_B^-m_{B-}^-$) *gal dcm rne131* (DE3)	Invitrogen Corp.
P. aeruginosa		
PAO1		
PAO1	Wild type	B. H. Holloway
Δ*brlR*	PAO1 Δ*brlR* (PA4878)	17
Δ*phoP*	PAO1 PW3128 *phoP*-F08::IS*lacZ*/hah	59
Δ*phoQ*	PAO1 PW3131 *phoQ*-C09::IS*lacZ*/hah	59
Δ*pmrA*	*pmrA*::*xylE*-Gm^r	45
Δ*pmrB*	*pmrB*::*xylE*-Gm^r	45
CF 1-2	*P. aeruginosa* isolate from CF patient	50
CF 1-2 Δ*brlR*	CF 1-2 harboring Δ*brlR* (PA4878); Gm^r	17
CF 1-8	*P. aeruginosa* isolate from CF patient	50
CF 1-8 Δ*brlR*	CF 1-8 harboring Δ*brlR* (PA4878); Gm^r	17
CF 1-13	*P. aeruginosa* isolate from CF patient	50
CF 1-13 Δ*brlR*	CF 1-13 harboring Δ*brlR* (PA4878); Gm^r	17
A1	*P. aeruginosa* isolate from CF patient	This study
A2	*P. aeruginosa* isolate from CF patient	This study
A7	*P. aeruginosa* isolate from CF patient	This study
C2	*P. aeruginosa* isolate from CF patient	This study
D5	*P. aeruginosa* isolate from CF patient	This study
Plasmids		
pJN105	Arabinose-inducible gene expression vector; pBRR-1 MCS *araC*-P_{BAD} Gm^r	31
pMJT1	Arabinose-inducible gene expression vector; pUCP18 MCS *araC*-P_{BAD} $Amp/Carb^r$	32
pJN-*bdlA*-His_6V5	C-terminal His_6V5-tagged *bdlA* cloned into pJN105 at EcoRI/SpeI	60
pJN-*brlR*	*brlR* cloned into pJN105	17
pMJT-*brlR*-His_6V5	C-terminal His_6V5-tagged *brlR* cloned into pMJT1	17
pMJT-*phoP*	*phoP* cloned into pMJT1	This study
pMJT-*phoQ*	*phoQ* cloned into pMJT1	This study

4-amino-arabinose to the lipid A moiety of LPS through the action of the *arnBCADTEF* operon. This modification reduces the negative charge of LPS, thus reducing the interaction between LPS and cationic antimicrobial peptides. In *P. aeruginosa*, activation of *arnBCADTEF* expression has been linked to two separate two-component regulatory systems, PhoPQ and PmrAB, which respond to limiting extracellular concentrations of divalent Mg^{2+} and Ca^{2+} (25–27). In contrast, peptide-mediated adaptive resistance requires the two-component systems (TCS) ParRS and CprRS, which lead to activation of the LPS modification system upon exposure to a wide range of antimicrobial peptides (28, 29).

Here, we asked whether BrlR contributes to the tolerance of *P. aeruginosa* biofilms to colistin, a cationic antimicrobial peptide. Inactivation of *brlR* correlated with increased resistance of biofilm cells to colistin, while overexpression of *brlR* resulted in increased susceptibility. The phenotype of susceptibility to colistin of *P. aeruginosa* overexpressing *brlR* correlated with reduced expression of *phoP*, *phoQ*, and *arnC* and was comparable to that of Δ*phoP*. Moreover, BrlR was found to directly bind to *oprH* promoter DNA of the *oprH-phoPQ* operon. Our findings establish BrlR as an unusual member of the MerR family of multidrug transport activators in that it acts as a repressor of PhoPQ activation and impairs tolerance of *P. aeruginosa* to colistin.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and culture conditions. All the bacterial strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* strain PAO1 was used as the parental strain. All planktonic cultures were grown in flasks at 220 rpm and 37°C using Vogel and Bonner citrate minimal medium (VBMM) (30), which contains the equivalent of 1 mM Mg^{2+} unless otherwise stated. Antibiotics were used at the following concentrations: 60 μg/ml tetracycline, 50 μg/ml gentamicin, and 250 μg/ml carbenicillin for *P. aeruginosa* and 20 μg/ml tetracycline, 20 μg/ml gentamicin, and 50 μg/ml ampicillin for *Escherichia coli*.

Strain construction. Complementation and overexpression of *brlR* and *phoP* were accomplished by inserting the respective genes in the pJN105 (31) or pMJT1 (32) vector under the control of an arabinose-inducible promoter. The plasmids were introduced into *P. aeruginosa* via conjugation or electroporation. Insertion was confirmed by PCR and sequencing. The primers used for strain construction are listed in Table S1 in the supplemental material.

Planktonic antibiotic susceptibility testing. To determine the role of BrlR in antimicrobial susceptibility to colistin, *P. aeruginosa* strains grown planktonicall to exponential phase y in VBMM at 37°C were treated with colistin (30 μg/ml) for 30 min and subsequently homogenized, serially diluted, and spread plated onto LB agar. Viability was determined via CFU counts. Susceptibility is expressed as log reduction.

MIC determination. The MIC of colistin was determined by performing a series of 2-fold dilutions in LB and VBMM containing 1% arabinose, using 96-well microtiter plates. The method was adapted from that of Andrews (33). Carbenicillin (250 μg/ml) or gentamicin (50 μg/ml) was added for plasmid maintenance. The antibiotic concentrations used ranged from 0.08 to 40 μg/ml for colistin and 0.09 to 100 μg/ml for tobramycin. The inoculum was ~10^4 cells per well, and growth inhibition was observed following overnight incubation at 37°C. The MIC was defined as the lowest antibiotic concentration that yielded no visible growth.

Biofilm formation. For biofilm antibiotic susceptibility testing, biofilms were grown under flowing conditions in a flow tube reactor system (1 m long, size 13 silicone tubing; flow rate, 0.1 ml/min; Masterflex; Cole Parmer, Inc.) with an inner surface area of 25 cm^2 (34–36). Biofilms were grown at 22°C in VBMM. To ensure overexpression of *phoP* and *brlR*, the respective strains were grown in the presence of 0.1% arabinose and 10 μg/ml carbenicillin or 2 μg/ml gentamicin.

Biofilm antibiotic susceptibility assays. Biofilms grown for 1 day under flowing conditions were treated for 2 h under flowing conditions with tobramycin (150 μg/ml) and colistin (100 μg/ml). Higher antibiotic concentrations were used than for planktonic susceptibility assays to account for the known fact that biofilms are more tolerant to antibiotics than their planktonic counterparts (6, 37, 38). Combination treatment was carried out by treating biofilms with both colistin and tobramycin at the concentrations mentioned above. Following exposure of biofilms to the respective antimicrobial agents, the biofilms were harvested by squeezing the tubing, followed by the extrusion of the cell paste as previously described (39). The resulting suspension was then homogenized, serially diluted, and spread plated onto LB agar. Viability was determined via CFU counts. Susceptibility is expressed as log reduction. Under the conditions tested, a total of 5×10^8 CFU were detected, on average, per biofilm tube reactor following 1 day of biofilm growth prior to treatment (see Fig. S1 in the supplemental material).

qRT-PCR. Isolation of mRNA and cDNA synthesis was carried out as previously described (36, 40–42). Quantitative reverse transcriptase PCR (qRT-PCR) was performed using the Eppendorf Mastercycler ep realplex (Eppendorf AG, Hamburg, Germany) and the KAPA SYBR Fast qPCR Kit (KAPA Biosystems, Woburn, MA), with oligonucleotides listed in Table S1 in the supplemental material. *mreB* was used as a control. The stability of *mreB* levels was verified by 16S RNA abundance using primers HDA1/HDA2 (43). Relative transcript quantitation was accomplished using the ep realplex software (Eppendorf AG) by first normalizing transcript abundance (based on the threshold cycle [C_t] value) to that of *mreB*, followed by determining transcript abundance ratios. Melting curve analyses were employed to verify specific single-product amplification.

Electrophoretic mobility shift assays. Assays of BrlR binding to the promoter region of the *oprH-phoPQ* operon were performed using the LightShift Chemiluminescent EMSA kit (Thermo Scientific), as previously described (44). Briefly, the biotinylated target DNA fragment P_{oprH} (+1 to +206 relative to the translational start site) was amplified using the primer pair oprH-prom-F/oprH-prom-R (see Table S1 in the supplemental material). A total of 0.05 pmol of target DNA was incubated for 30 min at room temperature with extracts obtained from *P. aeruginosa* overexpressing His_6V5-tagged BrlR in 25 mM Tris-Cl, pH 8, 5 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 mM EDTA, and 50 ng/μl poly(dI-dC) as nonspecific competitor DNA. A total of 1.25 to 10 pmol of His_6V5-tagged BrlR (as indicated by immunoblot analysis using purified His_6V5-tagged BrlR) was used. Extracts obtained from *P. aeruginosa* harboring the empty vector (pMJT1) and a Δ*brlR* mutant were used as negative controls. DNA gel shift assays were also carried out using extracts obtained from *E. coli* overexpressing His_6V5-tagged BrlR. For specific competition, nonbiotinylated target DNA (0.05 to 0.5 pmol) was used. Samples were separated on a 5% native polyacrylamide-Tris-borate-EDTA (TBE) gel, blotted onto a Hybond nylon membrane, and probed with anti-biotin antibodies, and bands were subsequently visualized according to the manufacturer's instructions (Thermo Scientific).

Statistical analysis. Student's *t* test was performed for pairwise comparisons of groups, and multivariate analyses were performed using 1-way analysis of variance (ANOVA), followed by an *a posteriori* test using Sigma Stat software.

RESULTS

Previous findings indicated the MerR-like transcriptional regulator BrlR plays a role in the high-level tolerance of *P. aeruginosa* biofilms to antimicrobial agents known to be substrates of multidrug efflux pumps (17). To determine whether BrlR contributes to the tolerance of *P. aeruginosa* to the cationic antimicrobial peptide colistin, MIC studies were carried out. The MIC is an indicator of the antimicrobial tolerance of a microorganism and is defined by its ability to grow in the presence of an elevated level of an antimicrobial agent.

TABLE 2 Overexpression of *brlR* in planktonic cells correlated with a 2-fold decrease in the MIC for colistin

Growth medium	MIC (μg/ml) of colistin for strain:				
	PAO1	Δ*brlR*	PAO1/pMJT-*brlR*	Δ*phoP*	Δ*phoQ*
LB	1.25	2.5	0.63	0.63	0.63–1.25
VBMM	2.5	5–10	0.63–1.25	0.63	1.25–2.5

[a] All strains were grown in VBMM containing 1.0% arabinose or LB medium.

MICs were determined by 2-fold serial dilutions in VBMM using 96-well microtiter plates and an inoculum of ~10^4 cells per well. The lowest antibiotic concentration yielding no visible growth for *P. aeruginosa* PAO1 was found to be 2.5 μg/ml (Table 2). Overexpression of *brlR* in *P. aeruginosa* (PAO1/pMJT-*brlR*) correlated with a colistin MIC decrease of up to 4-fold, while *brlR* inactivation rendered *P. aeruginosa* more tolerant to colistin, as evidenced by a 4-fold increase in the MIC up to 10 μg/ml (Table 2). Consistent with the observation that limiting concentrations of extracellular divalent cations, such as Mg^{2+}, contribute to antimicrobial peptide resistance in *P. aeruginosa* (26, 45, 46), no difference in the MIC was noted between the wild-type and Δ*brlR* strains when VBMM containing high Mg^{2+} concentrations (20 mM instead of 1 mM Mg^{2+}) was used (data not shown). However, in the presence of high Mg^{2+} concentrations (20 mM), overexpression of *brlR* correlated with a 2-fold reduction in the colistin MIC (data not shown). The effect of a lower Mg^{2+} concentration on the colistin MIC was not tested, as under these conditions (e.g., 10 μM Mg^{2+}), reduced growth rates were observed. The MIC of colistin for *P. aeruginosa* PAO1 was also determined in LB. The MIC for *P. aeruginosa* PAO1 was found to be 1.25 μg/ml. Under the conditions tested, inactivation or overexpression of *brlR* had a less pronounced effect on the colistin MIC than VBMM (Table 2). Compared to that of the wild type, the MIC of colistin for the strain expressing *brlR* (PAO1/pMJT-*brlR*) was 2-fold lower. Inactivation of *brlR* correlated with a 2-fold increase in the colistin MIC in *P. aeruginosa* (Table 2). While previous reports indicated *brlR* expression was biofilm specific (17, 18), the observed effect of *brlR* inactivation on the colistin MIC suggests *brlR* is expressed under planktonic conditions, with the medium composition (LB or VBMM versus VBMM-20 mM Mg^{2+}) contributing to *brlR* expression. The findings are in agreement with the detection of *brlR* transcripts under planktonic conditions by Wurtzel et al. (47) using RNA-Seq.

Given the marked difference in MICs between LB medium and VBMM, in particular with respect to differential *brlR* expression (Table 2), all subsequent experiments were carried out using VBMM. To further explore the role of BrlR in colistin resistance, we next determined the susceptibility to colistin of *P. aeruginosa* cells grown planktonically to exponential phase. On average, 8.9×10^9 CFU/ml was detected prior to treatment for *P. aeruginosa* PAO1, the Δ*brlR* strain, and a strain overexpressing *brlR* (Fig. 1A). Inactivation of *brlR* rendered *P. aeruginosa* grown planktonically more resistant to colistin (30 μg/ml, equivalent to 10 times

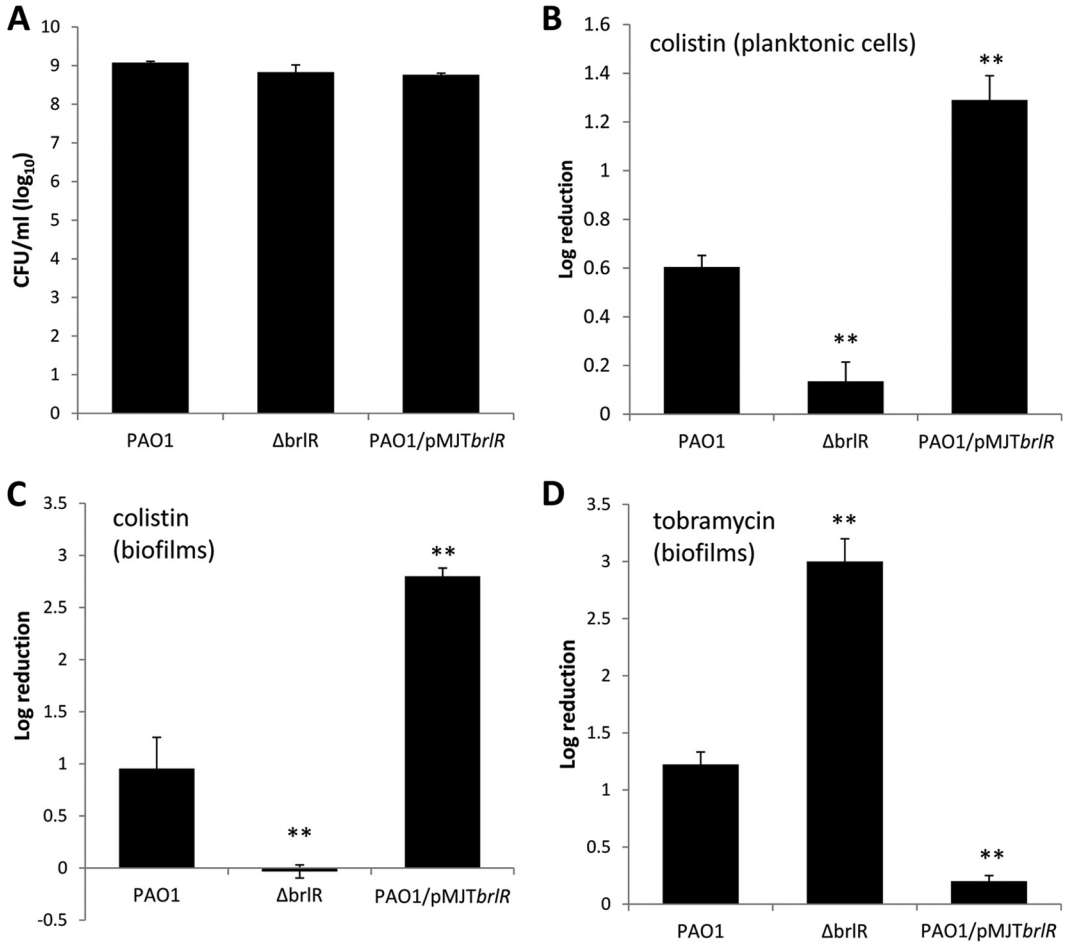

FIG 1 Inactivation of *brlR* renders biofilms more susceptible to tobramycin but resistant to colistin. (A) Total CFU obtained from *P. aeruginosa* strains grown to exponential phase in VBMM prior to treatment. (B) Susceptibilities to colistin (30 μg/ml) of exponential-phase-grown *P. aeruginosa* wild type and strains with *brlR* inactivated or overexpressing *brlR*. Susceptibility was determined by viable counts (CFU) and is expressed as log reduction. (C and D) Susceptibilities of *P. aeruginosa* wild-type biofilms and biofilms of strains with *brlR* inactivated or overexpressing *brlR* to colistin (100 μg/ml) (C) or tobramycin (150 μg/ml) (D). Biofilms were grown in tube reactors for 1 day under flowing conditions using VBMM as the growth medium. The biofilms were treated with colistin or tobramycin for 2 h under flowing conditions. Susceptibility was determined by viable counts (CFU). Experiments were carried out at least in triplicate. The error bars denote standard deviations. **, significantly different from the values for *P. aeruginosa* PAO1 ($P \leq 0.01$).

the MIC for wild-type PAO1) than the wild type (Fig. 1B). In contrast, overexpression of *brlR* rendered exponential-phase planktonic cells more susceptible to colistin, as indicated by the increase in log reduction for PAO1/pMJT-*brlR* compared to PAO1 (Fig. 1B).

Considering the effects of *brlR* inactivation and *brlR* overexpression on colistin resistance under planktonic growth conditions, we next asked whether BrlR also plays a role in the resistance of *P. aeruginosa* biofilms to colistin. As biofilms are more tolerant to antibiotics than their planktonic counterparts (6, 37, 38), *P. aeruginosa* wild-type and mutant biofilms with *brlR* inactivated or overexpressing *brlR* were treated longer and with higher concentrations of colistin (40 times the colistin MIC [100 μg/ml] noted for the wild type) than for planktonic susceptibility assays. Treatment of wild-type biofilms with colistin for 2 h resulted in an ~1-log-unit reduction in viability, while no viability reduction was observed for Δ*brlR* mutant biofilms (Fig. 1C). In contrast, overexpression of *brlR* rendered biofilms significantly more susceptible than wild-type biofilms (Fig. 1C).

The findings suggest that BrlR contributes to colistin resistance of *P. aeruginosa*, probably by altering the MIC. However, it is interesting that the contribution of BrlR to colistin resistance was opposite to the previously described role of BrlR in the high-level tolerance of *P. aeruginosa* biofilms to antimicrobial agents. While inactivation of *brlR* rendered *P. aeruginosa* more resistant to colistin, previous results suggested that *brlR* inactivation coincided with biofilms being more susceptible to bactericidal antibiotics, including tobramycin and norfloxacin (17, 18). Considering that the latter observations were made using LB-grown rather than VBMM-grown biofilms, we next asked whether the different contributions of BrlR to tobramycin resistance in *P. aeruginosa* are due to differences in the growth media. Similar to previous results (17), however, Δ*brlR* mutant biofilms were more susceptible to tobramycin, whereas biofilms overexpressing *brlR* were more resistant than the wild type (Fig. 1D).

Expression of *brlR* inversely correlates with expression of *arnC*, *phoP*, *phoQ*, *pmrA*, and *pmrB*. To begin elucidating the mechanism by which BrlR modulates colistin resistance in *P.*

TABLE 3 Fold change in transcript levels of genes linked to colistin resistance relative to PAO1 biofilms

Strain	Fold change in transcript levels relative to PAO1 biofilms[a]				
	arnC	*phoP*	*phoQ*	*pmrA*	*pmrB*
Δ*brlR*	5.4 ± 1.5	4.4 ± 0.5	7.3 ± 1.5	14.9 ± 0.8	27.8 ± 2.2
PAO1/pMJT-*brlR*	−1.75 ± 0.1	−2.1 ± 0.2	−2.8 ± 0.1	−1.6 ± 0.06	−1.6 ± 0.06

[a] All the strains were grown as biofilms, and qRT-PCR analysis was carried out in triplicate. Standard deviations are indicated.

aeruginosa, we asked whether BrlR contributes to the expression of the *arnBCADTEF* operon (26, 45, 46, 48). Compared to *P. aeruginosa* wild-type biofilms, Δ*brlR* mutant biofilms were characterized by a 5.4-fold increase in *arnC* transcript abundance, while overexpression of *brlR* correlated with decreased *arnC* expression, as determined using qRT-PCR (Table 3). In *P. aeruginosa*, four TCS have been shown to act upon the *arn* operon, namely, ParRS and CprRS, which are activated upon sensing antimicrobial peptides, and PmrAB and PhoPQ, which respond to limiting extracellular concentrations of divalent Mg^{2+} and Ca^{2+} (25–29). qRT-PCR confirmed that inactivation of *brlR* correlates with increased *phoP* and *phoQ* expression compared to wild-type biofilms, while decreased *phoP* and *phoQ* transcript abundance was detected in biofilms overexpressing *brlR* (Table 3). Likewise, expression levels of *pmrA* and *pmrB* were upregulated 15- and 28-fold, respectively, in Δ*brlR* biofilms compared to wild-type biofilms (Table 3). The findings are in agreement with previous transcriptomic data indicating that *brlR* inactivation coincides with a 45-fold increase in *arnC* expression in biofilms and correlates with increased expression of *phoP* and *phoQ*, as well as *pmrA* and *pmrB*, compared to the wild-type biofilms (18). As no difference in *parR*, *parS*, *cprR*, and *cprS* expression was noted upon *brlR* inactivation by transcriptomic analysis (18), these systems were not pursued further. Our findings indicate that BrlR contributes to the repression of *arnC*, *phoP*, *phoQ*, *pmrA*, and *pmrB* transcript abundance, with absence of BrlR coinciding with increased transcript levels.

Inactivation of *phoP* and *phoQ* renders *P. aeruginosa* biofilms susceptible to colistin. To determine whether PmrAB and PhoPQ contribute to the observed resistance of *P. aeruginosa* biofilms to colistin in a BrlR-dependent manner, we next tested the susceptibilities of mutant biofilms with *phoP*, *phoQ*, *pmrA*, and *pmrB* inactivated to colistin using 40 times the MIC. It is interesting that *phoP* and *phoQ* are included in an operon with *oprH*. Macfarlane et al. (48) previously demonstrated that OprH does not contribute to colistin resistance, and therefore, we did not test an *oprH* mutant. Compared to wild-type biofilms, no significant difference in viability was noted in Δ*pmrA* and Δ*pmrB* mutant biofilms (Fig. 2A). The finding suggested that at the colistin concentration tested, the TCS PmrAB likely plays no role in the resistance of *P. aeruginosa* biofilms to colistin. In contrast, inactivation of *phoP* rendered biofilms significantly more susceptible to colistin than wild-type biofilms (Fig. 2B). The susceptibility of Δ*phoP* mutant biofilms to colistin was comparable to the log reduction observed for biofilms overexpressing *brlR* (Fig. 1C). While the biofilm architecture of *phoP* mutant biofilms differed from that of wild-type biofilms (see Fig. S2 and Table S2 in the supplemental material), no significant difference in the biofilm CFU between wild-type and *phoP* mutant biofilms was noted (see Fig. S1 in the supplemental material). Complementation of Δ*phoP* mutant biofilms with *phoP* restored the resistance phenotype to wild-type levels (Fig. 2B). Moreover, Δ*phoQ* mutant biofilms were more susceptible to colistin than the wild type but were less susceptible than Δ*phoP* mutant biofilms (Fig. 2B). The resistance of Δ*phoQ* to colistin was restored to wild-type levels by complementation with *phoQ* (Fig. 2B).

The observed difference in susceptibility to colistin between Δ*phoP* and Δ*phoQ* mutant biofilms was also apparent in the MIC assays (Table 2). While the MICs for a Δ*phoP* mutant strain showed a 2-fold decrease in the colistin MIC in LB medium and up to a 6-fold

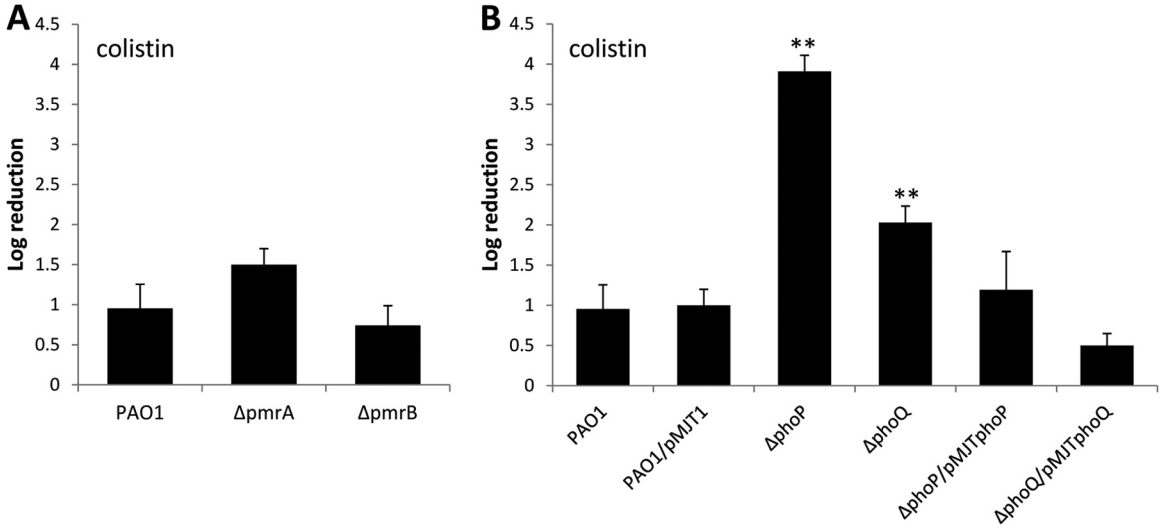

FIG 2 Inactivation of *phoP* and *phoQ*, but not *pmrA* or *pmrB*, renders biofilms more susceptible to colistin. Biofilms grown for 1 day in biofilm tube reactors were treated for 2 h with colistin (100 μg/ml) under flowing conditions. Susceptibility was determined by viable counts (CFU). (A) Susceptibility of *P. aeruginosa* wild-type biofilms and biofilms of strains with *pmrA* and *pmrB* inactivated to colistin (100 μg/ml). (B) Susceptibility of *P. aeruginosa* wild-type biofilms, Δ*phoP* and Δ*phoQ* biofilms, and mutant biofilms complemented with *phoP* and *phoQ in trans* to colistin. Experiments were carried out at least in triplicate. The error bars denote standard deviations. **, significantly different from the values for *P. aeruginosa* PAO1 ($P \leq 0.01$).

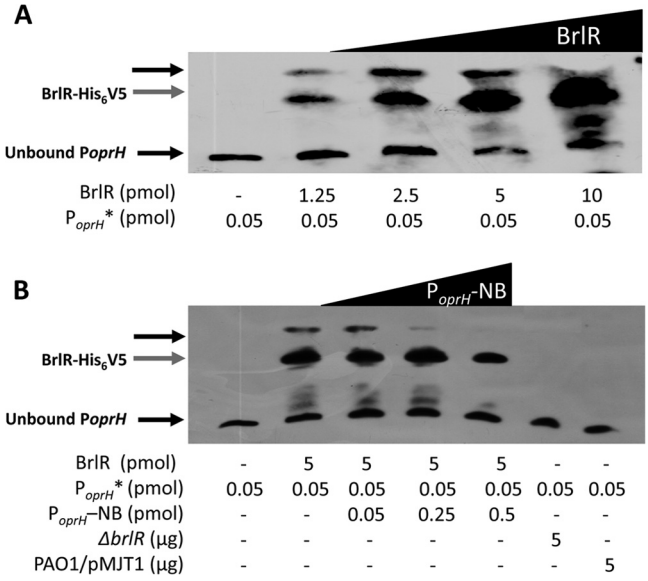

FIG 3 BrlR binds to the promoter of the *oprH-phoPQ* operon. Shown are DNA gel mobility shift assays using cell extracts obtained from *P. aeruginosa* expressing His_6V5-tagged BrlR (PAO1/pMJT-*brlR*-His_6V5) *in trans*. Both the concentration of His_6V5-tagged BrlR (1.25 to 10 pmol) (A) and the concentration of unlabeled competitor DNA (P_{oprH} -NB; 0 to 0.5 pmol) (B) were varied. Cell extracts obtained from *P. aeruginosa* harboring the empty vector (PAO1/pMJT1) and a Δ*brlR* mutant were used as negative controls. The P_{oprH} DNA fragment is 208 bp long, and 0.05 pmol of the biotinylated DNA fragment was used (P_{oprH} *). BrlR binding to P_{oprH} was detected by immunoblot analysis using anti-biotin antibodies. The band for unbound P_{oprH} is visible at the bottom of each image. The gray arrows indicate unbound His_6V5-tagged BrlR. The black arrows near the top of the image indicate shifts. Experiments were carried out in triplicate, and representative images are shown.

decrease in VBMM compared to the wild type, inactivation of *phoQ* only correlated with a 2-fold reduction in the colistin MIC.

BrlR binds to the promoter region of the *oprH-phoPQ* operon. Our findings suggested PhoPQ, but not PmrAB, contributes to the resistance of *P. aeruginosa* biofilms to colistin, with inactivation of *phoP* rendering biofilms as susceptible to colistin as *brlR* overexpression (Fig. 1C and 2B). Taking into account that multicopy expression of *brlR* coincided with reduced transcript abundances of *phoP* and *phoQ* and the PhoPQ-dependent gene *arnC* (Table 3), we hypothesized that BrlR is either directly or indirectly involved in repression of these transcripts. To determine whether the *phoPQ* operon is a direct target of BrlR, electrophoretic mobility shift assays were carried out using His_6V5-tagged BrlR (BrlR-His_6V5) and biotinylated promoter DNA, as previously described (18). BrlR DNA binding was dependent on both the BrlR and *oprH* promoter DNA concentrations. When the molar concentrations of BrlR were increased, binding to P_{oprH} increased (Fig. 3A). When increasing amounts of the unlabeled specific competitor P_{oprH} fragment were added to the gel mobility shift assay mixture, decreased BrlR binding to the labeled fragment was observed (Fig. 3B). Similar results were obtained when *E. coli* extracts overexpressing His_6V5-tagged BrlR were used (see Fig. S3 in the supplemental material). These results indicated that BrlR binds to the promoter region of the *oprH-phoPQ* operon. Taken together with the gene expression results, our findings indicate that BrlR is involved in transcriptional regulation of the *oprH-phoPQ* operon, acting as a repressor.

BrlR contributes to colistin resistance through PhoPQ. We next hypothesized that if BrlR acted upon colistin resistance only through PhoPQ, expression of *brlR* in a Δ*phoP* mutant would not render the mutant more susceptible. To address this question, we generated a Δ*phoP* mutant strain overexpressing *brlR*. While complementation of the Δ*phoP* strain with *phoP* restored the resistance phenotype of Δ*phoP* biofilms to wild-type levels, multicopy expression of *brlR* in did not render Δ*phoP* biofilms more susceptible to colistin (Fig. 4A). Moreover, *brlR* expression did not significantly alter the biofilm architecture of Δ*phoP* biofilms (see Fig. S2 and Table S2 in the supplemental material). However, plasmid-borne expression of both *brlR* and *phoP* resulted in reduced sus-

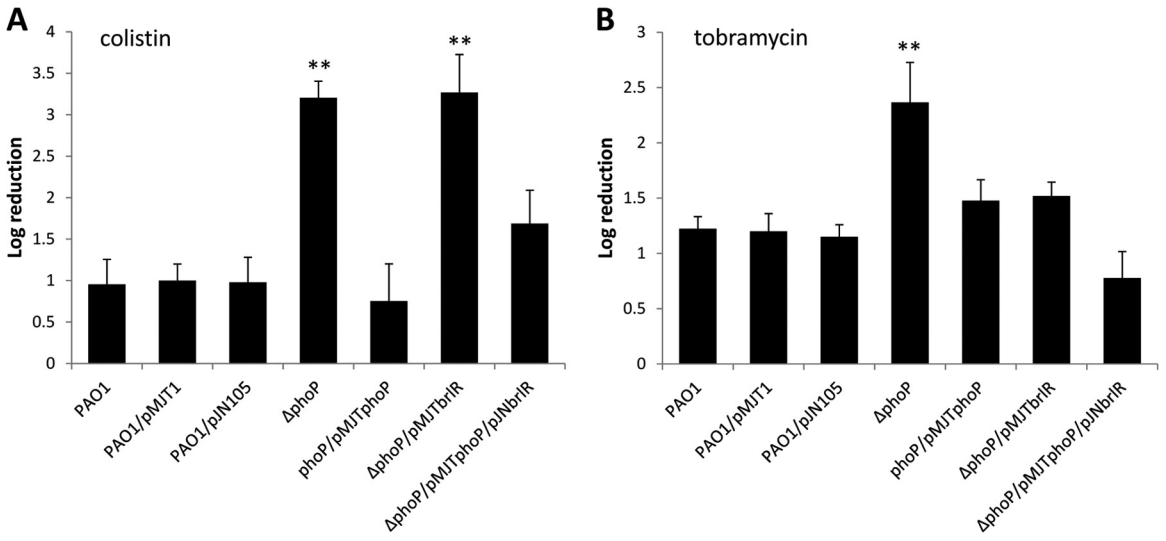

FIG 4 Expression of *brlR* in Δ*phoP* mutant biofilms has no effect on resistance to colistin. Biofilms of *P. aeruginosa* PAO1 and Δ*phoP* and complemented Δ*phoP* mutant strains (Δ*phoP*/pMJT*phoP* and Δ*phoP*/pMJT*phoP*/pJN*brlR*) were grown in VBMM for 1 day under flowing conditions and subsequently treated with colistin (100 μg/ml) (A) and tobramycin (150 μg/ml) (B). Susceptibility was determined by viable counts (CFU). Experiments were carried out at least in triplicate. The error bars denote standard deviations. **, significantly different from the values for *P. aeruginosa* PAO1 ($P \leq 0.01$).

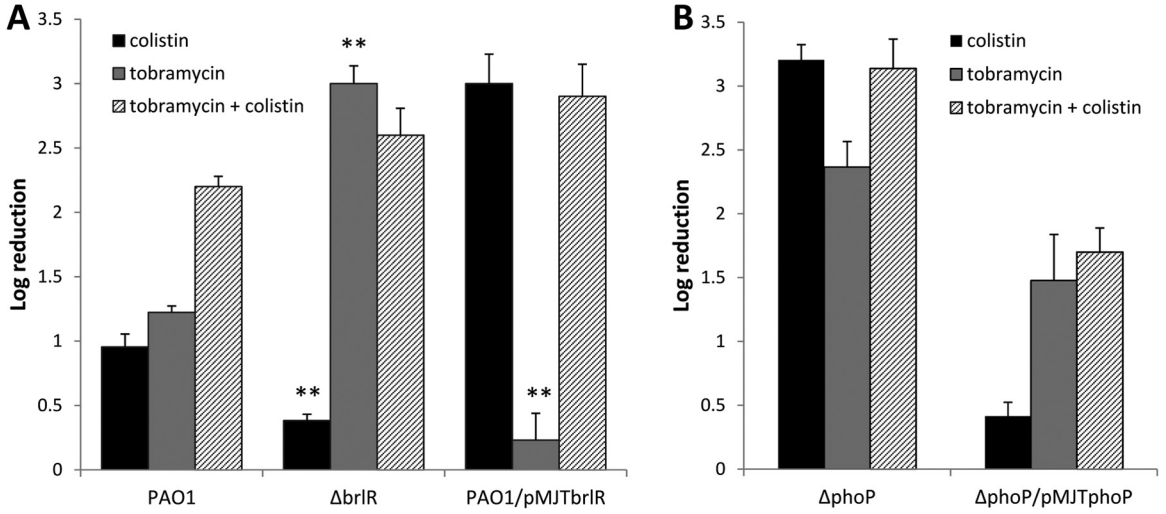

FIG 5 BrlR contributes to the resistance to colistin and tobramycin by *P. aeruginosa* PAO1 biofilms in a reciprocal manner. The susceptibilities of *P. aeruginosa* wild-type biofilms and biofilms with *brlR* inactivated or overexpressing *brlR* to 100 μg/ml colistin, 150 μg/ml tobramycin, or a combination of 150 μg/ml tobramycin and 100 μg/ml colistin were determined. One-day-old biofilms were treated for 2 h under flowing conditions. **, significantly different from the values for *P. aeruginosa* PAO1 under the same treatment ($P \leq 0.01$). (B) Susceptibilities of Δ*phoP* biofilms and Δ*phoP*/pMJT-*phoP* biofilms following exposure to 100 μg/ml colistin, 150 μg/ml tobramycin, or a combination of 150 μg/ml tobramycin and 100 μg/ml colistin for 2 h under flowing conditions. The experiments were carried out at least in triplicate. The error bars denote standard deviations.

ceptibility of Δ*phoP*/pMJT*phoP*/pJN*brlR* biofilms compared to Δ*phoP* (Fig. 4A). No difference in susceptibility was noted for the vector controls (Fig. 4A). The findings indicated that BrlR-dependent susceptibility to colistin of *P. aeruginosa* biofilms required PhoP. While multicopy expression of *brlR* in the Δ*phoP* strain did not alter the susceptibility of Δ*phoP* biofilms to colistin, Δ*phoP* biofilms overexpressing *brlR* were more resistant to tobramycin than Δ*phoP* biofilms (Fig. 4B). Our data are in agreement with previous reports indicating a role of PhoPQ in aminoglycoside resistance under planktonic growth conditions (25, 49). Complementation restored the resistance phenotype of the Δ*phoP* strain to wild-type biofilm levels (Fig. 4B). Similar results were obtained for Δ*phoP* biofilms coexpressing *phoP* and *brlR* (Fig. 4B).

BrlR contributes to colistin and tobramycin resistance in a reciprocal manner. Overall, our findings strongly support a dual nature of BrlR with respect to biofilm resistance, as overexpression of *brlR* resulted in enhanced susceptibility to colistin but reduced susceptibility to tobramycin (and the opposite upon *brlR* inactivation). Considering the reciprocal contribution of BrlR to colistin and tobramycin resistance, we next hypothesized that *P. aeruginosa* biofilms are equally susceptible to dual treatment with colistin and tobramycin regardless of the level of *brlR* expression.

Biofilms overexpressing *brlR* were equally susceptible to colistin alone and colistin together with tobramycin but resistant to tobramycin alone (Fig. 5A). Similarly, combination treatment rendered Δ*brlR* biofilms significantly more susceptible than did treatment with colistin alone (Fig. 5A). The susceptibility to colistin and tobramycin noted for Δ*brlR* biofilms was comparable to that observed following tobramycin treatment. Moreover, treatment with both antibiotics had an additive effect for *P. aeruginosa* PAO1 biofilms, which were found to be overall more susceptible to tobramycin and colistin than to treatment with either antibiotic alone (Fig. 5A). Similar results were obtained for complemented Δ*phoP* mutant biofilms (Fig. 5B). In contrast, however, no significant difference in susceptibility was noted when Δ*phoP* mutant biofilms were treated with either colistin or tobramycin alone or in combination (Fig. 5B). Our data suggested that while differential *brlR* expression affected the resistance of *P. aeruginosa* when treated with colistin or tobramycin alone, dual treatment with colistin and tobramycin alleviated the effect of *brlR* expression levels on resistance and resulted in superior killing of *P. aeruginosa* wild-type biofilms. Moreover, our findings indicate that enhanced killing observed upon dual treatment is based on the differential expression of *brlR*, but not *phoP*.

Expression of *brlR* renders CF clinical isolates more susceptible to colistin but more resistant to tobramycin. To further explore the reciprocal contribution of BrlR to colistin and tobramycin resistance, we made use of two sets of clinical isolates, with each set originating from a single cystic fibrosis patient. Set 1 comprised isolates A1, A2, A7, C2, and D5, while set 2 comprised isolates CF 1-2, CF 1-8, and CF 1-13. Analysis of the three cystic fibrosis isolates by Southern blotting using a 741-bp PstI-NruI fragment, derived from the upstream region of exotoxin A, demonstrated that the three isolates originated from the same parental strain (50). Whether the isolates comprising set 1 are derived from the same parental strain is unknown. However, numerous studies have shown that isolates obtained from an individual cystic fibrosis patient typically originate from a single parental strain (51–54), and it is therefore likely that the set 1 isolates are clonal. It is interesting that native *brlR* expression in these clinical isolates grown under surface-associated conditions was comparable to *brlR* transcript levels observed in *P. aeruginosa* biofilms as determined using qRT-PCR (see Table S3 in the supplemental material). The only exceptions were isolates CF 1-2 and CF 1-13, which were previously shown to have 4- and 8-fold-increased *brlR* transcript levels, respectively, compared to wild-type biofilms (17) (see Table S3 in the supplemental material). We next conjugated the plasmid pJN-*brlR* into each clinical isolate and subsequently determined the MICs of colistin and tobramycin for clinical isolates and clinical isolates expressing *brlR* from *P. aeruginosa*

TABLE 4 MICs of colistin and tobramycin for clinical isolates[a]

Clinical isolate[b]	MIC (μg/ml) of colistin			MIC (μg/ml) of tobramycin		
	-	pMJT-*brlR*	Δ*brlR*	-	pMJT-*brlR*	Δ*brlR*
A1	5	2.5		6.25	25	
A2	2.5	1.25		6.25	50	
A7	0.6	0.3		12.5	25	
C2	≥40	5		12.5	50	
D5	0.6	0.15		3.1	50	
CF 1-2	2.5	0.3–0.6	2.5	3.1	12.5	1.56
CF 1-8	1.25	0.6	1.25	1.56	6.25	1.56
CF 1-13	20	0.6	≥40	50	≥100	1.56

[a] Multicopy expression of *brlR* resulted in a decrease in the colistin MIC but an increased tobramycin MIC for cystic fibrosis clinical isolates, while inactivation of *brlR* coincided with an increased colistin MIC but a decreased tobramycin MIC. MIC assays were carried out using LB. The values are based on a minimum of three experiments.
[b] CF isolates A1, A2, A7, C2, and D5 were obtained from P. Singh; strains CF 1-2, 1-8, and 1-13 were originally described by Ogle et al. (50).

PAO1. A total of 8 clinical isolates were thus tested. As shown in Table 4, the MICs for colistin and tobramycin varied significantly among the clinical isolates tested. For instance, isolate C2 was found to have a colistin MIC of 40 μg/ml and a tobramycin MIC of 12.5 μg/ml, while isolate D5 was characterized by lower MICs of both, 0.625 μg/ml for colistin and 3.125 μg/ml for tobramycin. However, while the MICs differed significantly among the clinical isolates tested, a shift in the MICs upon plasmid-borne expression of *brlR* was observed in each of the clinical isolates. For clinical isolate C2, expression of *brlR* resulted in a 6-fold decrease in the MIC for colistin but a 4-fold increase in the MIC for tobramycin. For isolate D5, the colistin-MIC decreased 4-fold while the tobramycin-MIC increased 8-fold. Similar changes in the MIC were observed for all other clinical isolates tested, with *brlR* expression resulting in a decreased colistin MIC but an increased tobramycin MIC (Table 4).

We also determined whether inactivation of *brlR* in a subset of clinical isolates correlated with a reciprocal shift in the MIC. To do so, we made use of three clinical isolates, namely, CF 1-2, CF 1-8, and CF 1-13. With the exception of CF 1-8, isolates with *brlR* inactivated demonstrated increased colistin MICs but decreased tobramycin MICs compared to the isogenic parental strains (Table 4). It is interesting that isolate CF 1-8 was shown to possess a nonsense mutation in the *brlR* gene, resulting in a truncated protein (17). The finding that *brlR* inactivation in CF 1-8 did not affect the MIC compared to the isogenic parental strain indicated that the truncation rendered BrlR nonfunctional. Our data suggest that BrlR contributes in a reciprocal manner to the tolerance to colistin and tobramycin, with BrlR conferring tolerance to tobramycin while rendering *P. aeruginosa* susceptible to colistin (and the opposite upon *brlR* inactivation).

To further explore whether differential expression of *brlR* from *P. aeruginosa* and its reciprocal contribution to colistin and tobramycin resistance can be overcome by combination treatment in clinical isolates, biofilms formed by isolates CF 1-2, CF 1-8, and CF 1-13 and isogenic mutant strains with *brlR* inactivated or overexpressing *brlR* were treated with tobramycin, colistin, and a combination of colistin and tobramycin. Susceptibility was determined by viability assays. Biofilms formed by isolates CF 1-2 and CF 1-13 were as resistant to tobramycin and colistin as *P. aeruginosa* PAO1 (Fig. 6A). Combination treatment had little effect on the susceptibility of CF 1-2 and CF 1-13 biofilms compared to treatment with either antibiotic alone (Fig. 6B and C). Inactivation of *brlR* in isolate CF 1-2 correlated with reduced killing by colistin relative to the parental strain (Fig. 6B). Dual treatment of CF 1-2 Δ*brlR* rendered biofilms of the isolate significantly more susceptible than did treatment with colistin alone. In contrast, *brlR* overexpression resulted in isolate CF 1-2 being more susceptible to colistin and combination treatment than the parental strain (Fig. 6B). Overall, dual treatment of CF 1-2 with *brlR* inactivated or overexpressing *brlR* was more effective than treatment of the parental strain (Fig. 6B). Similar results were obtained for isolate CF 1-13 with *brlR* inactivated or overexpressing *brlR* compared to the parental strain (Fig. 6C). The reciprocal contribution of BrlR to colistin and tobramycin resistance was less pronounced in biofilms formed by clinical isolate CF 1-8, likely because CF 1-8 harbors a nonsense mutation in the *brlR* gene (17). Thus, biofilms of CF 1-8 and CF 1-8 Δ*brlR* were found to be equally susceptible to colistin but significantly more susceptible to tobramycin than *P. aeruginosa* PAO1 biofilms (Fig. 6A and D). However, similar to multicopy expression of *brlR* in CF 1-2 and CF 1-13, overexpression of *brlR* in CF 1-8 biofilms rendered the strain more susceptible to colistin than the parental strain, while combination treatment had an intermediate effect on antibiotic susceptibility (Fig. 6C). These observations are in agreement with the MIC data obtained for this isolate (Table 4).

Taken together, the data strongly support a role of BrlR in reciprocally contributing to biofilm susceptibility to colistin and tobramycin, with dual treatment overcoming the difference in susceptibility due to differential *brlR* expression.

DISCUSSION

A hallmark of biofilms is their profound tolerance to antimicrobial agents. In addition to previously described mechanisms contributing to the antibiotic tolerance of biofilms, including slow growth and diffusion limitation, *P. aeruginosa* biofilms also employ a specific regulatory mechanism involving the transcriptional regulator BrlR to resist the action of antimicrobial agents known to be multidrug efflux pump substrates (17). In particular, BrlR has been demonstrated to contribute to biofilm tolerance to norfloxacin, tobramycin, chloramphenicol, tetracycline, and trimethoprim by activating the expression of at least two multidrug resistance pumps, thus affecting the MIC and recalcitrance to killing by bactericidal antimicrobial agents (17, 18). However, the protective role of BrlR appears to be absent in *P. aeruginosa* biofilms with respect to the cationic antimicrobial peptide colistin. Instead, overexpression of *brlR* resulted in increased susceptibility to colistin, with BrlR acting as a repressor of *phoPQ* expression while inactivation of *brlR* made these mutants substantially more resistant. Expression of the *phoPQ* operon is activated in response to limiting extracellular concentrations of divalent Mg^{2+} and Ca^{2+} (25–27) and, outside of autoregulation of the *oprH-phoPQ* operon by PhoP itself (26), little is known about the regulation of this TCS. The finding that BrlR functions as a repressor of *phoPQ* gene expression provides a novel level of control of the regulation of the PhoPQ TCS. The PhoPQ system in *P. aeruginosa* is unique compared to more thoroughly characterized homologs. Unlike the *Salmonella* PhoPQ system, which directly or indirectly regulates over 200 genes, the *P. aeruginosa* PhoPQ regulon comprises fewer than 20 genes (26, 55). Moreover, while the *Salmonella* PhoPQ acts through PmrAB to regulate downstream gene expres-

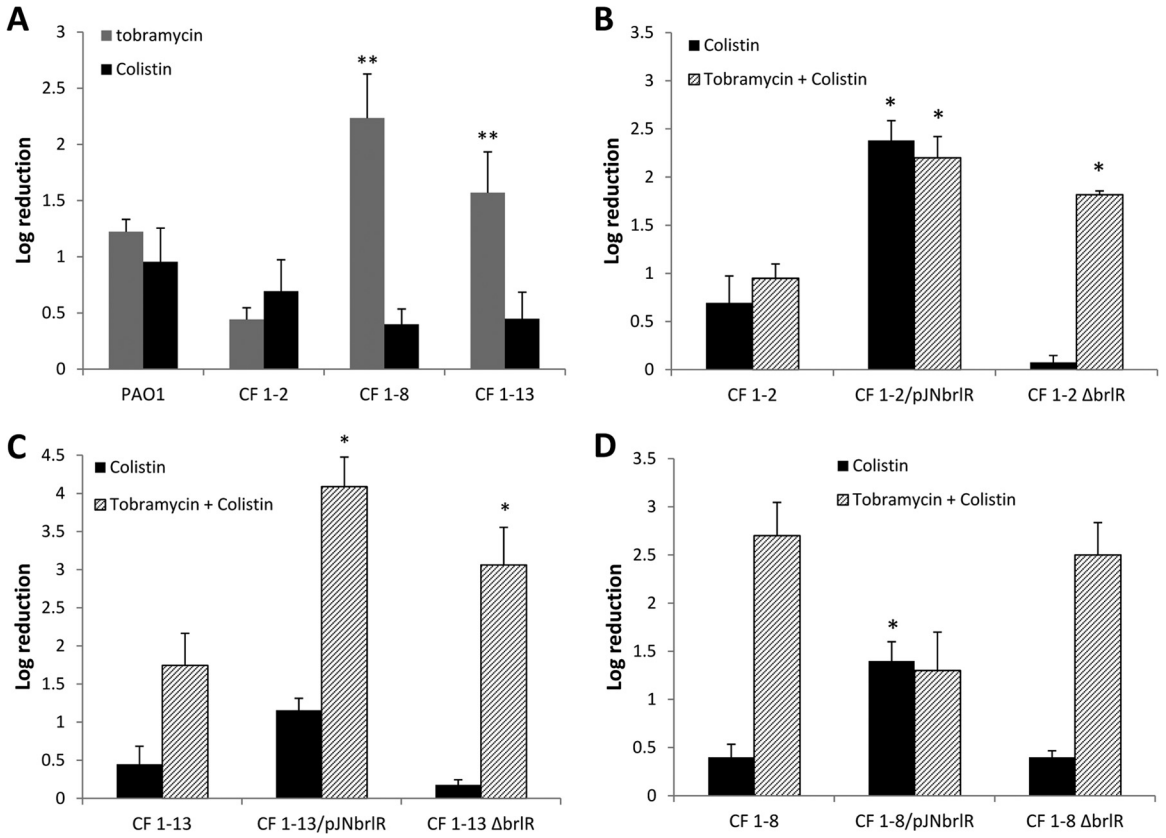

FIG 6 BrlR contributes to resistance to colistin and tobramycin by *P. aeruginosa* cystic fibrosis isolates in a reciprocal manner. (A) Susceptibility determination of *P. aeruginosa* PAO1 and clinical CF isolates CF 1-2, CF 1-8, and CF 1-13 grown as biofilms (1 day). The biofilms were treated with 150 μg/ml tobramycin or 100 μg/ml colistin for 2 h under flowing conditions. **, significantly different ($P \leq 0.01$) from PAO1. (B to D) Determination of susceptibilities of 1-day-old biofilms formed by isolates CF 1-2, CF 1-8, and CF 1-13 and isogenic strains with *brlR* inactivated or overexpressing *brlR*. The biofilms were treated with 100 μg/ml colistin or a combination of 150 μg/ml tobramycin and 100 μg/ml colistin for 2 h under flowing conditions. *, significantly different from the values for the parental strain ($P \leq 0.01$). Experiments were carried out at least in triplicate. The error bars denote standard deviations.

sion, evidence suggests that in *P. aeruginosa*, PmrAB activation is independent of PhoPQ (26, 56), and both PhoPQ and PmrAB activate the expression of the *arnBCADTEF* operon responsible for the addition of 4-amino-arabinose to LPS (26, 45). However, under the conditions tested, no evidence of PmrAB contributing to the resistance of *P. aeruginosa* biofilms to colistin was noted in this study. Instead, only PhoPQ was found to be required for biofilm resistance to colistin. To our knowledge, this is the first description of PhoPQ mediating colistin resistance in *P. aeruginosa* biofilms. Thus, while PmrAB likely acts independently of PhoPQ under planktonic conditions, our observations suggest that under biofilm growth conditions, PmrAB either plays no role in colistin resistance or, alternatively, that PhoPQ and PmrAB do not act independently in *P. aeruginosa* biofilms.

Cationic antimicrobial peptides, including colistin, are frequently used in the treatment of pulmonary lung infections in CF patients colonized by *P. aeruginosa* (57). Previous findings suggested colistin-tobramycin combination treatments result in increased killing of *P. aeruginosa* biofilms compared to treatment with either antibiotic alone (58). Here, we likewise observed enhanced killing of *P. aeruginosa* PAO1 biofilms and biofilms of clinical isolates CF 1-2 and CF 1-13 upon dual treatment compared to monotherapy. While it is unclear why BrlR would directly or indirectly repress genes responsible for resistance to antimicrobials such as colistin while contributing to tolerance to antibiotics that are multidrug efflux pump substrates (17, 18), the reciprocal role of BrlR in repressing colistin resistance while enhancing resistance to tobramycin provides a genetic basis for the superior killing by tobramycin and colistin when used in combination.

In summary, we demonstrate that in *P. aeruginosa*, resistance to the cationic antimicrobial peptide colistin is reciprocally regulated by PhoPQ and the transcriptional regulator BrlR. Moreover, our findings demonstrate that BrlR is an unusual member of the MerR family of multidrug efflux pump activators. While members of this family have been demonstrated to function solely as activators, BrlR functions as both an activator of muldtidrug efflux pump genes (18) and a repressor of PhoPQ activation.

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